Frozen Jaguar *(Panthera onca)* sperm capacitation and ability to penetrate zona free hamster oocytes

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Abstract

Assisted reproductive technologies in endangered species, such as artificial insemination, in vitro fertilization and embryo transfer, can be viewed as one potential approach for safeguarding species. Toward this aim, the objective of this study was to evaluate the fertility of frozen jaguar (Panthera onca) sperm and Tyrod's Talp PVA capacitation medium using the hamster zona free oocyte penetration assay. Ejaculates were collected from nine animals using electroejaculation and cryopreserved. Sperm capacitation was performed by swim-up technique using Tyrod's Talp PVA medium at room temperature. Penetration was considered when the spermatozoa head decondensation was visualized within the oocyte. This assay showed 15.4 % penetrations (350/2275 oocytes). Results of this study showed high sperm abnormalities, low sperm quality after cryopreservation, and low percentage of penetrations. However, the penetration results showed that the cryopreserved jaguar's semen can be used for artificial insemination, in vitro fertilization and intra cytoplasmatic sperm injection, supporting the semen bank creation for this specie.

Key words:
Panthera onca.
Penetration assay.
Sperm capacitation.
Sperm cryopreservation.

Introduction

The jaguar (*Panthera onca*), the largest wild cat of the Americas, is currently listed in the Apendix I of the International Convention of Endangered Species[1] and in the Red List of Threatened Species/IUCN as Near Threatened[2]. The jaguar's population has declined rapidly due to deforestation and depletion of prey base as result of human development.

Considering the importance of the propagation in captivity to the preservation of endangered species, jaguar's reproduction in captivity needs to become an integral component for conservation this species. Captive populations of nondomestic felids are valuable as educational, scientific and genetic resource that can be used to support conservation of wild cats.[3,4,5]

Assisted reproduction is becoming an increasingly important tool in the genetic management of these species.[6,7,8,9] However, successes in application of assisted reproduction techniques on wildlife cats have been sparse. If gamete cryostorage is to be practical for assisting in the propagation of nondomestic species, it must be determined whether freeze-thawing compromises sperm function.

This study assessed the ability of frozen-thawed jaguar spermatozoa to bind, and penetrate heterologous oocytes in vitro, after capacitation. Capacitation is a biochemical process that occurs on the sperm cell surface, which enables the spermatozoa to undergo the acrosome reaction before fertilization.[10] The penetration assay in zona free hamster oocyte indirectly indicate capacitation by measuring the ability of spermatozoa to penetrate zona free hamster oocytes.[3,11]

The objective of this study was to evaluate the fertility of frozen jaguar sperm and capacitation medium Tyrod's Talp PVA using the hamster zona free oocyte penetration assay.

Material and Method

Animals

Nine adult (10 - 12 years old) male jaguars, were housed in outdoor pens at the Fundação Parque Zoológico de São Paulo, São Paulo, SP (n = 2), Parque Zoológico Municipal "Quinzinho de Barros", Sorocaba, SP (n = 2) and Bosque dos Jequitibas, Campinas, SP (n = 5), and maintained on diets of red meat (beef) and organ meat (bovine heart) with vitamin and mineral supplement (Rhoster Ltda.).

Semen collection, evaluation and cryopreservation

Animals were anaesthetized (10 mg/kg tiletamine-zolazepam; Zoletil, Virbac, Brazil) by blow dart. Semen was collected by electroejaculation.[12,13] A rectal probe (diameter, 2.4 cm; length 29 cm) and electroejaculator (AC, 60Hz sine-wave; Eletrovet, São Paulo, SP, Brazil) were used. Eighty electrical stimuli were administered, divided in three series (30:30:20) in the following manner: serie 1 (ten stimuli at 2 v, ten at 3 v and ten at 4 v), serie 2 (ten stimuli at 3 v, ten at 4 v and ten at 5 v) and serie 3 (ten stimuli at 5 v and ten at 6 v). Samples were transported to the laboratory at 37°C and semen was immediately evaluated for total volume and pH (reagent strips - Sigma). Motility (0 – 100 %) and rate of forward progression (vigor: 0 - 5) were based on observations of four separated microscopic fields at 40x. An aliquot was diluted (1 : 3) in a 10 % formaldehyde saline solution and used to determine the sperm concentration using a Neubauer chamber. Sperm morphology evaluations were performed after fixing in a formaldehyde saline solution and examining 100 individual sperm cells using phase contrast microscopy (1000 x).[12]

Each ejaculate was diluted (1:1) in HEPES solution (Sigma, H-0887) and centrifuged (300 g for 10 min). After centrifugation, the supernatant was removed and the aliquot was resuspended in PDV 62 in two steps[14]: first using an A cryodiluent (20 % egg yolk, 11 % lactose, 1000 IU/ml penicilin, 1000 mg/ml streptomicin) was added slowly and mixed gently with the sperm pellet at room temperature. The mixture was equilibrated for 1 h at 5°C. After this time a B cryodiluent (20 % egg yolk, 11 % lactose, 1000 IU/ml penicillin, 1000 mg/ml streptomycin and 8 % glycerol), at 5°C, was added slowly and mixed gently with the sperm pellet.[14] After addition of glycerol the spermatozoa suspension was allowed to equilibrate at 5°C for 30 min after transfer to cooled 0.25 ml straws. One end of each straw was tapped with polivinilic alcohol and placed on liquid nitrogen vapor for 20 min, immersed in liquid nitrogen, transferred to rack and loaded into canes for storage in liquid nitrogen.

Zona free hamster oocytes penetration assay

Mature unfertilized oocytes were collected from the oviducts of 60 superovulated hamster (*Mesocrisetus auratusa*) 15 to 17 h after an intraperitoneal injection of 35 IU human chorionic gonadotropin – hCG (Vetecor 5000UI, Serono) and 72 h after an intraperitonial injection of 35 IU pregnant mare serical gonadotropin - PMSG (Intergonan 1000UI Vemie Veterinar Chemie). The oocyte were free from the surrounding cumulus cells by treating them with 1 mg/ml hyaluronidase[15] (Sigma, H-3506) in HEPES solution (Sigma, H-0887), them treated for 1 or 2 min with 0.1 % trypsin (Sigma, T-8253) in HEPES solution (Sigma, H-0887) to remove the zona pellucida.[16]

Straws of semen were removed from liquid nitrogen and thawed immediately in a 39°C waterbath for 1 min and evaluated (motility and vigor). Contents of the straws were transferred to 15 ml centrifuge tubes with Talp PVA medium (1 : 1) and centrifuged at 700 g for 10 min. The

supernatant was discarded and the pellet resuspended to a concentration of 0.05 x 10^6 spermatozoa / ml in Tyrod's medium and maintained at room temperature for 30 min before insemination.[17]

Fifteen zona free oocytes were placed in 100 ml of Talp PVA medium under mineral oil in a plastic Petri dish (Corning, 60 x 15mm) and 25 ml sperm suspension was additioned. The preparation was incubated at 38°C for 3 h in 5 % CO_2 in air.

The ability of spermatozoa to penetrate oocyte was assessed by glutaraldehyde fixation and acetic orcein staining in slides visualized in phase contrast microscopy. Oocytes were considered as penetrated when the spermatozoa head decondesation was visualized within the oocyte.[18]

Statistical Analysis

For the statistical analysis the Student t test (SSPS 9.0 for Windows, SSPS Incorporation, Chicago, Illinois) was utilized.

Results

Semen collection technique was highly efficient and 100% of the procedures produced an ejaculate. Urine contamination occurred in 15% of ejaculates. Physical and morphological characteristic of the ejaculates are presented in table 1. Analysis pre cryopreservation showed a high percentage of semen abnormalities (72.4 ± 4.3%) (Table 1). The abnormalities were classified as primary defects and secondary defects. Primary defects were as follows: macrocephalic, bicephalic, abnormal midpiece, no midpiece, biflagellate, abnormal head shape, abnormal acrosome, microcephalic, pear-shaped head, spear-shaped head, abnormal acrosome and tightly coiled tail. Secondary defects were as follows: bent midpiece with droplet, bent tail with droplet, bent neck; bent midpiece without droplet, bent tail without droplet and proximal droplet.

There were significant differences ($p < 0.05$) between motility/vigor pre and pos cryopreservation (Table 2). A total 2275 oocytes was analysed and 350 showed sperm heads into the cytoplasm (15.4 %) (Table 3).

Table 1 - Jaguar's semen analysis, São Paulo, 2000

Animals	Volume (ml)	Motility (%)	Vigor (1-5)	pH	Concentr. (x 10^6/ml)	Normal (%)	Abnormal (%)
1	6.0	70	4	8.0	1.2	39	61
2	6.5	60	4	8.0	1.8	47	53
3	6.0	70	3	8.5	0.2	18	82
4	5.1	50	3	8.5	1.0	42	58
5	5.0	70	3	7.5	0.6	18	82
6	7.0	80	5	7.5	6.0	24	76
7	9.8	80	4	8.5	1.5	8	92
8	8.0	80	4	7.5	0.9	24	76
9	10.0	70	3	7.0	1.0	28	72
M / SE	7.0 ± 0.6	70 ± 3.3	3.7 ± 0.2	7.9 ± 0.1	1.6 ± 0.6	27.6 ± 4.3	72.4 ± 4.3

Table 2 - Jaguar's semen analysis pre and pos cryopreservation, São Paulo, 2000

Animals	Pre		Pos	
	Motility (%)	Vigor (1-5)	Motility (%)	Vigor (1-5)
1	70	4	20	3
2	60	4	20	3
3	70	3	20	4
4	50	3	20	3
5	70	3	20	2
6	80	5	20	3
7	80	4	20	3
8	80	4	50	3
9	70	3	50	4
M/SE	70 ± 3.3 [a]	3.7 ± 0.2 [c]	26.7 ± 4.4 [b]	3.1 ± 0.2 [d]

[a] and [b] significantly different, $p < 10^{-4}$ ($p = 1.98x10^{-5}$)
[c] and [d] significantly different, p = 0,018

Discussion

Eletroejaculation was a suitable method for semen collection in this threatened species; all collections were successful and were performed without problems. No change in animal's behavior or health was observed after collections, suggesting that the protocol used was clinically and ethically acceptable.

Sperm morphology evaluation in an ejaculate is used to characterize the number of normal and abnormal cells. Primary defects result from problems during spermatogenesis and secondary defects during final spermatozoa maturation in the epididymis.[19] Semen quality observed here was similar to previous studies.[5,20] A high percentage (mean 51%) of morphologically abnormal spermatozoa was present in the ejaculate of all males in a study of jaguars in Brazil.[20]

The presence of secondary defects could be related to poor manipulation of the ejaculate. In the present study, however, the collections were made in an appropriate manner, in accordance with the standard protocol.

Primary defects originate during spermatogenesis and may be the result of genetic, environmental or nutritional factors.[21] Poor quality of semen in wild felids has been associated with low genetic variability.[22] In a study in Latin American zoos, large felids maintained on nutritionally adequate diets had a lower percentage of normal sperm.[4] An analysis of genetic variability and/or paternity may contribute to the elucidation of the cause of low spermatic quality in jaguars in Brazilian zoos.

Recent findings from a Brazilian study support the need to improve the quality of enclosures in order to have a positive effect on reproductive success in small cats.[23] According to standards outlined in the Neotropical Feline Handling Plan[24], which determines the basic requirements for neotropical felids, the enclosures at all the participating zoos were inadequate in terms of structure and environment.

Several authors have associated a vitamin A deficiency with sperm alterations

Table 3 - Total oocytes, number of penetrations and % of jaguar's frozen semen penetration in zona free hamster oocytes, São Paulo, 2000

Animals	Total	Penetrated	% Penetrations
1	625	72	11.5 %
2	628	88	14.0 %
3	373	65	17.5 %
4	199	40	20.0 %
5	126	35	28.0 %
6	117	31	26.5 %
7	148	11	7.5 %
8	09	04	44.0 %
9	50	04	8.0 %
Total	2275	350	15.4 %

and a reduction in spermatogenesis.[5,25,26,27,28] A return to normal spermatogenesis after restorations of adequate supply of vitamin A has been recorded. In the same way, degenerating alterations in the testicles may be related to vitamin E deficiency.[29] This deficiency may cause irreparable damage to the testicles and even after re-establishment of the vitamin E supply, no improvement in sperm morphology occurred.[30]

Types of sperm abnormalities also varied significantly among species, serious head anomalies were predominant in the jaguar and midpiece malformations were more common in the puma.[4] The causes of specific morphology defects in cat sperm have not been identified, however, it is well known that these structurally deformed sperm do not participate in fertilization.[31]

Studying normospermic and teratospermic cats were observed that normospermic cats penetration was significant by higher than teratospemic cats.[32] In teratospermic spermatozoa of domestic cats a low penetration in zona free oocytes penetration assay was demonstrated.[12,33]

These studies indicated that teratospermy has a detrimental influence on sperm function and oocyte penetration. This suggests the low rate of penetration in the jaguar could be related to the high rate of morphological abnormal spermatozoa observed in the samples examined.

In domestic cats; penetrated cat oocytes did not decreased with capacitation at room temperature.[34] Capacitation at room temperature was observed and no preincubation time in CO_2 incubator was required for spermatozoa from the ductus deferents of cat to penetrate cat oocytes.[35]

In penetration assay of frozen jaguar sperm in heterologous oocytes using swim-up technique with Talp medium and 1 hour preincubation at CO_2 incubator determining 0 % penetration, by the other hand, preincubation at room temperature determining 8.1 % penetration.[17]

The tiger (*Panthera tigris*) frozen semen penetrations in zona free hamster oocytes without preincubation in CO_2 incubator was $7,6 \pm 5,6$ % and after 2 hours preincubation was 0 %.[36]

After thawing, spermatozoa may exhibit substantial acrosome damage but still have adequate viability to fertilize oocytes in vitro[14,37] or produce offspring with artificial insemination[38]. These findings are very important to the biology of conservation, since the grade of the teratospermy in

endangered wild felines has been shown to be very high and the creation of a gamete bank would be the solution to the future of numberless endangered felines.

Using the Student t test, we concluded that the motility and vigor average pre and pos cryopreservation were significantly different ($p < 0.05$). However, despite the high index of abnormalities and the damages caused by the freezing process, the spermatozoa were able to penetrate in 15.4 % of the oocytes.

Assisted reproduction techniques in wild animals, such as in vitro fertilization (IVF), have been considered to be the solution to the problems of sexual incompatibility and to the introduction of genetic material in isolated populations. Teratospermy might be one of the most important factors that influence the IVF efficiency in cats. However, the data indicates that ejaculates containing abnormal spermatozoa can be used to produce embryos.

In conclusion the swim-up technique using Tyrod's-Talp PVA medium at room temperature can be used for jaguar's sperm capacitation and penetration of frozen jaguar's sperm in hamster zona free oocyte penetration assay.

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Capacitação espermática de sêmem congelado de onça pintada (*Panthera onca*) e ensaio de penetração em oócitos de hamster livres de zona pelúcida

Resumo

Biotecnologias reprodutivas aplicadas a espécies selvagens, como inseminação artificial, fertilização *in vitro* e transferência de embriões, são vistas como um potencial caminho para proteção das espécies ameaçadas de extinção. Devido a isso, o objetivo deste estudo foi avaliar a fertilidade do sêmen congelado de onças pintadas (*Panthera onca*) e o meio de capacitação espermática usando o ensaio de penetração em oócitos de hamster livres de zona pelúcida. Ejaculados de nove animais foram coletados por eletroejaculação e criopreservados. Para determinar a capacitação espermática foi utilizada a técnica swin-up com meio Tyrods Talp PVA a temperatura ambiente. No ensaio de penetração em oócitos de hamster livres de zona pelúcida foram considerados como oócitos penetrados aqueles que apresentaram em seu interior a cabeça do espermatozóide descondensada. O ensaio foi realizado em um total de 2275 oócitos, dos quais 350 apresentaram em seu interior a cabeça do espermatozóide descondensada, perfazendo um total de 15.4% de penetração. Os resultados deste estudo demonstraram alto índice de anormalidades espermáticas, baixa qualidade do sêmen e baixa porcentagem de penetrações. Entretanto, os resultados de penetração espermática demonstraram que sêmen congelado de onça pintada poderá ser utilizado para inseminação artificial, fertilização *in vitro* e injeção intracitoplasmática dando suporte para a criação de um banco de sêmen para esta espécie.

Palavras-chave:
Panthera onca.
Ensaio de penetração.
Capacitação espermática.
Criopreservação de sêmen.

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